

Experimental and Theoretical Analysis to Assess the Use of Monolithic Columns in Process Chromatography

Simone Dimartino^a, Omon M Herigstad^b, Cristiana Boi^{*c}, Eleonora Lalli^c, Giulio Sarti^c

^a Present address: Department of Chemical and Process Engineering, University of Canterbury, Private Bag 4800, 8140 Christchurch, New Zealand.

^b Present address: Abbvie Bioresearch Center Inc., 100 Research Drive, Worcester, MA 01605, USA

^c Dipartimento di Ingegneria Civile, Chimica, Ambientale e dei Materiali, DICAM, Università di Bologna, via Terracini 28, 40131 Bologna, Italy.
cristiana.boi@unibo.it

Monolithic materials are novel and attractive supports to be used as stationary phase in chromatographic columns to be used in downstream processes. As microporous membranes, they operate in convective mode which is useful to overcome the diffusive limitations of conventional packed bed columns.

In this work, the adsorption and elution of human IgG onto convective interaction media (CIM) Protein A monolithic columns has been studied. Complete chromatography cycles, including adsorption, washing and elution, have been experimentally performed at several operating conditions and the results are discussed in detail. The frontal analysis of characteristic points (FACP) approach has been successfully applied for the first time for monolithic media to determine the dynamic binding isotherm for human IgG. Elution was performed over several operating conditions to determine the effect of pH and flow rate on the total recovery of IgG, and on the concentration of the eluted fraction.

1. Introduction

The primary capture of monoclonal antibodies by Protein A affinity-chromatography packed beds is the preferred method used by the biopharmaceutical industry. However, this operation suffers from several limitations such as high material and operational costs, diffusion as the primary transport phenomenon and difficulties associated with packing and scale-up. The employment of convective-based chromatographic media, like membranes and monoliths, could help to circumvent these operational limitations due to an appreciable decrease in pressure drops, increased mass transport rates, ease of packing and overall reduced processing times.

As first pointed out by Etzel (2003) and recently confirmed by Podgornik and Lendero Krajnc (2012), monoliths are particularly attractive for the isolation and purification of large biomolecules, for which, due to a higher accessible surface area, they display greater binding capacity than chromatographic beads. Several large molecules of interest have been successfully isolated and purified, like viruses, virus like particles, nucleic acids and plasmid DNA, demonstrating the advantages of monolithic supports for these applications.

Monoliths are also used in polishing steps of large scale antibody manufacturing, but in negative chromatography mode, where binding capacity for impurities is not an issue and the exploitation of convective dominated mass transport can significantly reduce processing times, as reported by Nascimento et al. (2014) among others.

The work presented here characterizes the transport phenomena, adsorption and elution of IgG in a commercially available convective interaction media (CIM) Protein A monolithic column for process-scale applications. A complete characterization of the CIM monolithic column was performed by Herigstad et al. (2015) using the moment analysis to determine the porosity and axial dispersion coefficient for several tracers covering a large range of molecular weights. The frontal analysis of characteristic points (FACP) approach has been successfully applied in measuring the isotherm of human IgG in a staircase mode as described by Guiochon et al (2006). To validate this method, the adsorption isotherm data were also obtained in dynamic

experiments performed at complete saturation over a wide range of experimental conditions. The bed utilization of the Protein A CIM disk was calculated and critically compared to literature data of a commercial Protein A packed bead column. Finally, a detailed study of elution was performed under several operating conditions to determine the effect of pH and flow rate on both the total recovery of IgG and the elution profile.

2. Materials and experimental methods

2.1 Materials

Gammanorm, a polyclonal human immunoglobulin G solution, was chosen as IgG source (Octapharma, Stockholm, Sweden). All other chemicals and proteins were purchased from Sigma-Aldrich (Milan, Italy).

Protein-A monolithic convective interaction media (CIM) were a kind gift of BIA Separations (Ljubljana, Slovenia). The CIM disks have a height of 3 mm, a diameter of 12 mm and a nominal pore diameter of 1.4 μm . Chromatographic runs were performed on a Fast Protein Liquid Chromatography (FPLC) AKTA Purifier 100 (GE Healthcare, Milan, Italy) equipped with a 150 mL Superloop.

0.1 M phosphate buffer saline, pH 7.4 (PBS) was used as the running buffer in the loading and washing steps, while the elution buffer was a 0.1 M glycine solution at different pH values.

2.2 Experimental methods

The frontal analysis of characteristic points (FACP) method in a staircase mode has been adopted in this work to determine the equilibrium binding capacity of human IgG to the Protein A CIM. IgG solutions were fed, at a constant flow rate of 2.1 mL/min, to the column containing one Protein A monolithic disk by sequentially increasing the IgG concentration from 0.29 to 2.06 mg/mL. In addition, a step-down of IgG concentration (i.e. reverse staircase) was performed to determine the corresponding amount of protein desorbed, if any, during the washing stage. Experiments under non-binding conditions were performed to obtain the system dispersion curve.

A second set of experiments was designed to investigate the influence of flow rate and feed concentration on dynamic binding capacity of human IgG to the Protein A CIM disk. Complete chromatographic cycles consisting of adsorption, washing and elution stages were carried out at different IgG concentrations from 0.59 to 2.38 mg/mL and at different flow rates from 0.3 to 3.0 mL/min corresponding to linear velocities from 15.9 to 159.2 cm/h. The adsorption step was continued until complete breakthrough, that is obtained when the column is saturated. The column was then washed until the absorbance of the effluent fell back to the baseline value, and elution of bound IgG was carried out with a 0.1 M glycine buffer at a pH 3.5. The monolithic disc was regenerated after every 4 cycles using 0.1 M NaOH solution as indicated by the manufacturer's instructions.

Additional sets of experiments were carried out in order to investigate the influence of the elution conditions on IgG recovery. First, a step-gradient elution scheme was employed to determine the critical pH value required to induce dissociation of the IgG from the Protein A CIM disk. The monolithic column was loaded to saturation with IgG under binding conditions and subsequently washed until the UV reading fell back the baseline value. Elution was then carried out using a step-gradient from pH 6.0 to 2.75 in 0.25 pH increments in 0.1 M Glycine. In subsequent studies, the adsorption and washing cycles were performed under similar operating conditions, namely 0.6 mL/min flow rate and 0.92 mg/mL IgG concentration in the feed, while elution was carried out under different flow rates (0.3, 0.6, 1.5 and 3.0 mL/min) and elution buffer's pHs (3.0, 3.5, 4.0 and 4.5). Since the main objective of these experiments is the study of the elution parameters, the Protein A column was only loaded to approximately 10% breakthrough in order to moderate the amount of IgG used.

3. Results

3.1 Adsorption isotherm

For the system under investigation, the equilibrium binding capacity of IgG solutions to the Protein A disk was determined through the frontal analysis in a staircase mode. This method allows to rapidly collect data at different protein concentrations in the feed and to determine the equilibrium binding isotherm using a small amount of protein material. A parallel experiment under non-binding conditions was performed in order to evaluate the deviation from the ideal step due to band broadening. The actual amount of bound IgG for each feed concentration (i.e. each step) was calculated by numerical integration of the area between the observed breakthrough curve under binding and non-adsorbing conditions. A typical UV profile obtained during the construction of the staircase isotherm is plotted in Figure 1a. The resulting equilibrium binding isotherm is shown in Figure 1b together with dynamic binding capacity data obtained in separate experiments performed up to complete saturation. As it can be observed from the data reported in Figure 1b, the two data sets are consistent one to each other indicating the reliability of the staircase method to determine the adsorption isotherm in a fast and convenient fashion. All data were fit to the Langmuir isotherm resulting in a maximum

binding capacity, $q_m = 7.20 \pm 0.15$ mg/mL, and equilibrium dissociation constant, $K_d = 0.11 \pm 0.02$ mg/mL. The CIM discs IgG binding capacity is significantly lower than those observed for various commercial Protein A resins which depends on the support used and range from 25 to 70 mg/mL as reported by several research groups including Hahn and et al. (2003), Mc Cue et al. (2003) and Natarajan and Zydny (2013). However, the CIM may still provide overall process improvement through increased throughput, as shown later.

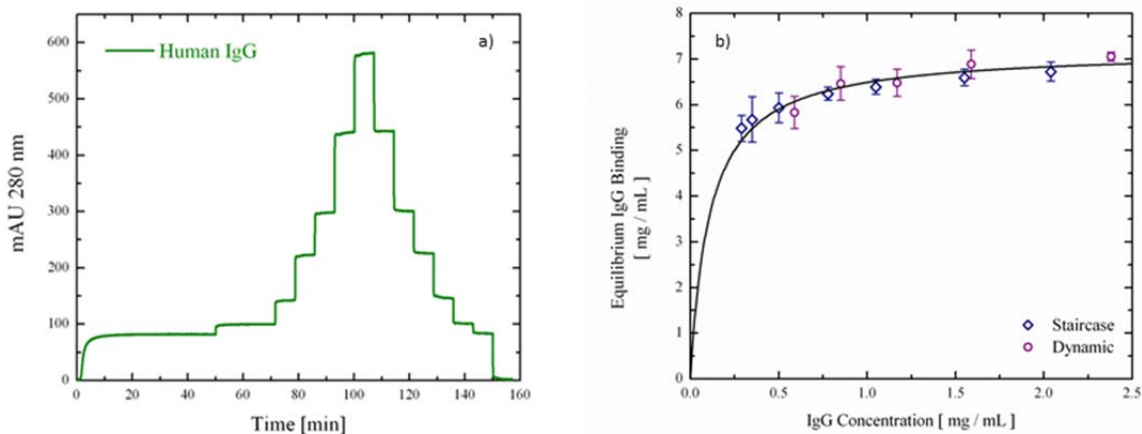


Figure 1 Adsorption isotherm of pure IgG at 4°C: absorbance profile as a function of time obtained during the construction of the staircase isotherm a); equilibrium isotherm with data obtained in the staircase mode and frontal analysis in dynamic mode up to saturation b).

3.2 Effects of flow rate on binding capacity

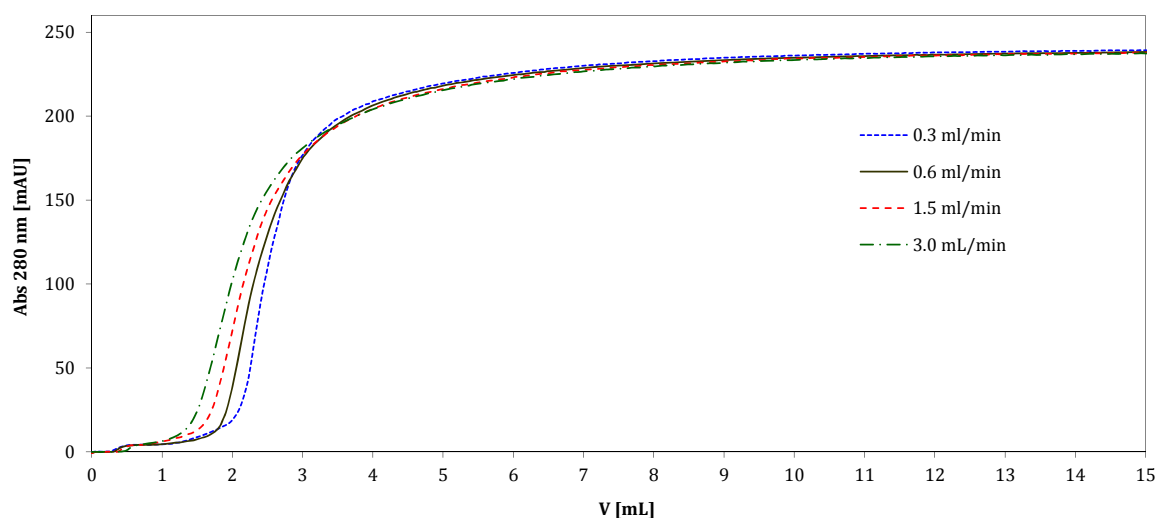


Figure 2 Effect of flow rate on the breakthrough curves in experiments performed at saturation by feeding solutions of 0.85 mg/mL of pure polyclonal IgG.

The effects of flow rate on the breakthrough curves can be observed in Figure 2 where the concentration profiles of IgG, obtained at a constant value of IgG in the feed, are plotted as a function of the total volume fed to the chromatographic system. At the onset of breakthrough, a small increase in the absorbance signal was recorded up to the first 1.5-2.0 mL of feed volume. That is likely due to the presence of IgG₃ in the feed solution, since the polyclonal Gammanorm feed contains 5% IgG₃, an IgG subclass that is weakly retained by Protein A, and therefore it exits immediately from the membrane module while the other subclasses are still adsorbed. Apart from the IgG₃ breakthrough, as it can be observed from the curves in Figure 2, a little, but visible effect of flow rate on binding capacity is present. This indicates that diffusional limitations are non-

negligible in the growing part of the breakthrough curve which is indeed relevant in most industrial applications. Since chromatographic separations are generally operated up to the breakthrough point, defined as the point in which the outlet protein concentration equals a small percentage of the inlet concentration, generally 10%, the effect of flow rate on binding capacity needs to be further investigated.

To this aim the bed utilization, defined as the ratio between the amount of IgG adsorbed at the breakthrough point and the maximum amount adsorbed at the same operating conditions, namely $DBC_{10\%}/DBC_{100\%}$, was calculated and the results compared with literature data for packed bead columns obtained by Hahn et al. (2005) and membrane adsorbers reported by Boi et al. (2008).

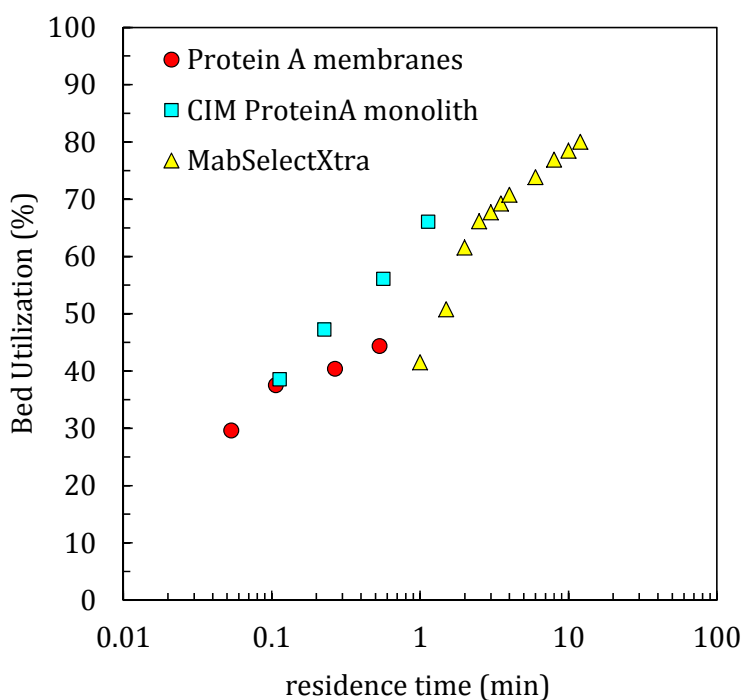


Figure 3 Comparison of bed utilization values for three different Protein A chromatographic supports: membranes, monoliths and beads.

As it is apparent from the data reported in Figure 3, the column packed with MabSelectXtra beads has the highest values of bed utilization with respect to the convective chromatographic supports. However, these data are associated to high residence times, that is at lower flow rates which correspond to long process times. At lower residence times, the bed utilization of CIM Protein A discs outperforms the results of MabSelectXtra beads and of membrane adsorbers. This suggests that the performance of monolithic columns for the capture step of monoclonal antibody deserve further investigation especially with scaled-up modules which can allow experiments at flow rates comparable to process conditions.

3.3 Elution study

A detailed study of elution was also undertaken to investigate the effects of pH and flow rate on the recovery of the bound IgG. The CIM column was loaded to 10% of the maximum binding capacity, washed to baseline, and then eluted at four different flow rates for each elution pH investigated. Figure 10 shows that the flow rate has little effect on the IgG elution profile for both the lower (Figure 10a) and upper (Figure 10b) range of elution pH investigated. This observation is consistent with a relatively fast elution kinetics not affected by the flow-rate, indicative that the characteristic time scale for elution is smaller than the time scale for axial dispersion and convection. Further quantitative considerations on the characteristic time scales are the subject of a separate study focusing on the adsorption and elution kinetics of IgG on the Protein A CIM disk.

The pH of the elution buffer was shown to have a very strong effect on both the recovery and eluted volume (i.e. concentration) of IgG. Figure 11 presents the IgG elution profiles at constant flow rate as a function of the four different pH values investigated, while Figure 12 shows the recovery (i.e. eluted IgG mass/bound IgG

mass) as a function of pH. It is apparent that the elution peaks broaden at higher pH, consistent with a drop in the desorption kinetics of the IgG from the affinity ligand. In particular, at pH 4.5 the elution profile is extremely shallow lasting more than 10 minutes with a maximum IgG concentration in the eluate in the order of 0.25 mg/mL, indicating suboptimal conditions for elution. On the other hand, at a low pH of elution becomes extremely sharp, with a narrow peak (< 1 mL) containing a highly concentrated solution of the target IgG (>8 mg/mL). The recovery is relatively constant between pH 3.0-3.5, and then drops precipitously with increasing pH. Extrapolation of data in Figure 12, at higher pH values, further suggests that the critical pH needed to initiate elution is in the range of pH 4.50-4.75, but it is not until pH 3.5–4 that full elution is triggered, confirming the results previously discussed in Figure 9. These considerations on the elution characteristics of the IgG molecule from the capture column are fundamental to identify the correct kinetic elution mechanism, which can then be employed for appropriate modelling for process analysis and scale-up purposes.

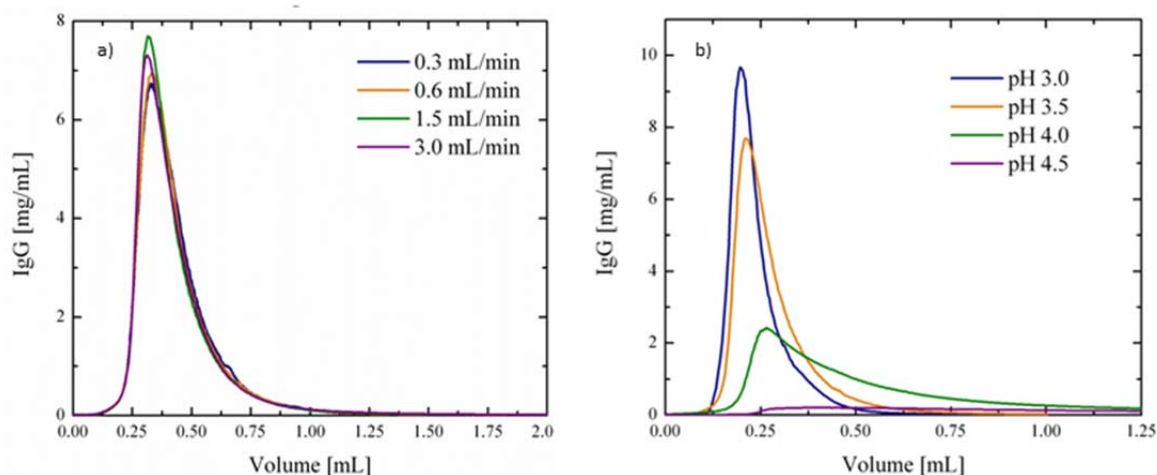


Figure 4 Effects of flow rate and buffer pH on elution of IgG: effect of flow rate in experiments performed at a constant pH of 3.5 a); effect of pH in experiments performed at a constant flow rate of 1.5 mL/min b).

4. Conclusions

A commercial Protein A affinity monolithic column has been completely characterized in view of its possible application for human IgG capture in a monoclonal antibody production process. The total porosity of 0.6 and the specific permeability of $5.74 \cdot 10^{-15} \text{ m}^2$ are comparable to literature data for CIM disks and different monolithic columns, and highlight that the surface area of the monolithic structure is fully available for binding of large biomolecules. Although the maximum binding capacity of 7.2 mg/mL, obtained with two different methods in experiments with polyclonal human IgG, is still very far from the binding capacity values of Protein A chromatography beads, the very short residence times and the higher bed utilization values are very encouraging for more economic productivity values and to promote research aiming at improved monolithic materials.

A detailed elution study has shown that, across the broad range of experimental condition examined, pH is the significant parameter that affects the elution profile and the IgG recovery, while the monolith response and recovery is practically insensitive to feed flow rate.

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